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Rapid Isolation of DNA from Chocolate and Date Palm Tree Crops

KENNETH M. HAYMES,[†] IBRAHIM A. IBRAHIM,[‡] SUE MISCHKE,*
DAVID L. SCOTT,[†,§] AND JAMES A. SAUNDERS[#]

Alternate Crops and Systems Laboratory, Plant Sciences Institute, Beltsville Agricultural Research Center, Building 50, Room 100, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland 20705

DNA isolation from plants is sometimes difficult due to the existence of high levels of endogenous phenolics, polysaccharides, or other substances that may interfere with DNA extraction. *Theobroma cacao* produces high levels of anthocyanins in young leaves. These plant polyphenols can interfere with DNA isolation. After examination of various procedures for DNA isolation, two commercial isolation procedures have proved to be repeatedly successful using these types of plants, the D^2 BioTechnologies DNA X-tract Plus kit and the Qiagen DNeasy Plant System DNA kit. These commercial kits were chosen for their speed and ease over the CTAB procedure, which is more labor intensive. All protocols assessed yielded DNA suitable for AFLP or SSR procedures. An additional factor in DNA extraction efficiency is the degree of cell breakage, which may be more difficult with the highly fibrous leaf tissue that is found in many monocots, including date palm. Two commercially produced pieces of equipment were tested and, for cacao, both resulted in template DNA yielding amplification product in AFLP or SSR fingerprinting. However, for the fibrous date palm leaf, the larger FastPrep homogenizer consistently yielded DNA that generated higher signals when amplified than did the smaller Disruptor Genie.

KEYWORDS: DNA fingerprinting; *Theobroma cacao*; *Phoenix dactylifera*; PCR; AFLP; SSR; microsatellite

INTRODUCTION

Screening of plant germplasm with molecular markers to characterize genetic diversity has become an important tool for gene mapping and breeding studies for economic crops (*1*−*3*). This is particularly true for tree crops, which involve long germination times between crosses. The need for a rapid DNA extraction procedure that works well in a variety of plants is paramount to this process. Although certain rapid DNA isolation procedures work well in some plants, it can be difficult to find simple procedures that are suited for a large variety of different plants (*4*). In this study, two tree crops with generation times of 6−8 years, which were difficult sources for DNA extraction, have been used as model systems to evaluate DNA isolation procedures. Each DNA sample was analyzed for the quantity and quality of DNA isolated using two different characterization methods, amplified fragment length polymorphism (AFLP) and single-sequence repeats (SSR) analysis. The AFLP procedure generally requires a higher quantity and quality of DNA than the SSR procedure, although both procedures will tolerate some non-DNA contamination of the preparations. The two crops used in this study were *Theobroma cacao* L. (cacao) and *Phoenix dactylifera* L. (date palm).

Chocolate is produced from beans, which are found in *T. cacao* pods. This crop faces major problems in tropical production areas, including variable or unknown plant varieties, disease pressure, and lack of resistant cultivars. Diseases of cacao, including witches' broom, frosty pod, and black pod, caused by fungal pathogens, have greatly affected cacao production and currently represent a serious threat to the cocoa industry in South America and in the Caribbean (*5*). Losses range from 30 to 70% in areas where cacao disease is endemic (*6*). Cultural practices such as pruning have not been sufficiently effective in minimizing damage, and the use of expensive chemical control methods is unsatisfactory due to high economic costs and environmental concerns. Furthermore, slow progress has been realized in traditional breeding programs aimed to release new cacao plant varieties that are uniform, highly productive, and tolerant to disease. A viable solution would be to employ molecular marker-assisted breeding strategies to identify economically important traits such as disease resistance, high yield, and quality of beans.

* Author to whom correspondence should be addressed [telephone (301) 504-5603; fax (301) 504-6478; e-mail mischkes@ba.ars.usda.gov].
[†] Permanent address: D-squared BioTechnologies Inc., P.O. Box 78853, Atlanta GA 30357.
[‡] Permanent address: Genetic Engineering and Biotechnology Research Institute (GEBRI), Menufia University, Sadat City, Egypt.
[§] Permanent address: Morehouse College, Atlanta, GA 30314.
[#] Permanent address: Director, Molecular Biology, Biochemistry and Bioinformatics, Towson University, 360 Smith Hall, 8000 York Road, Towson, MD.

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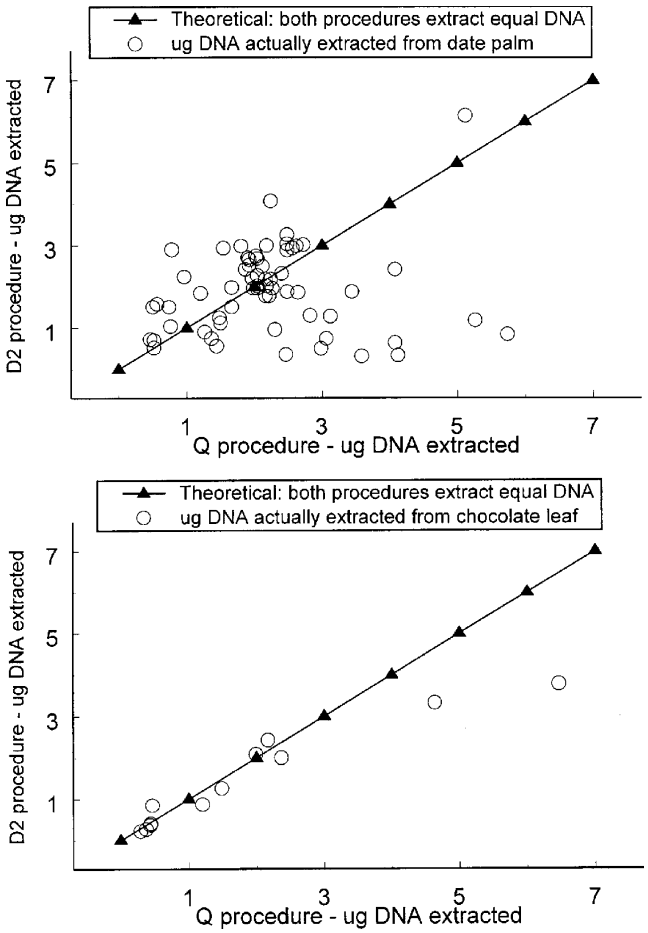

Figure 1. Comparison of DNA amounts isolated from equivalent samples by two procedures, D² BioTechnologies DNA X-tract Plus versus Qiagen DNeasy: (**A**) DNA was isolated from 62 date palm accessions, and (**B**) DNA was isolated from 12 leaf samples of cacao (*T. cacao*). The theoretical line represents identical extraction efficiencies of DNA by either procedure. Data above the line represent higher yields obtained with the D² BioTechnologies protocol, whereas points below the line represent higher quantities obtained with the Qiagen technique. No statistical difference was determined between observed and expected lines.

P. dactylifera (date palm) is a vital crop, widely grown in arid regions of North Africa and the Middle East where the tree is a basic component of the oasis. It grows in an environment that supports very few other crops. The fruit of the date palm is used as a staple for human consumption as well as for animal feed. Date palm breeding is hampered by long generation cycles of the trees. It typically takes almost 30 years to complete a successful hybridization and three backcrosses. The improvement of date palm varieties has not been approached using molecular breeding tools, although in vitro propagation of selected varieties is beginning to play an important role in the breeding process. Most of this effort has been directed toward a program to identify resistance to Bayoud, a fungal disease caused by *Fusarium oxysporum* Schlechtend Fr. *f.* sp. *albedinis* (*7*).

The use of Polymerase Chain Reaction (PCR) procedures as a molecular screening tool has yielded a number of strategies for the identification of economically important traits in various crops. DNA marker analysis techniques such as random amplified polymorphic DNA (RAPD), SSR, or the related procedure inter-simple sequence repeat (ISSR), sequence tagged microsatellite site (STMS), and AFLP have all been successfully employed for the selection of parents for conventional breeding and hybridization programs (*2*, *8*). However, the recovery of high-quality DNA represents one of the limiting steps in utilizing PCR-based molecular marker technology.

The methods previously employed for the isolation of DNA from cacao plants resulted in DNA contaminated with polysaccharides and phenolic compounds that required additional purification by ultracentrifugation on a CsCl density gradient (*9*−*11*). Time-consuming methods such as these make high-throughput analysis difficult. For our purposes, a DNA isolation method was needed to evaluate a large number of plants for a breeding program. We describe homogenization of leaf tissue and protocols using commercially available kits for the routine isolation of high-quality DNA from cacao and date palm. The resulting DNA is amenable to sensitive molecular marker technologies.

MATERIALS AND METHODS

DNA Isolation. DNA was isolated, with replication, according to four different methods from 50-mg leaf samples of the 12 *T. cacao* (chocolate) and 62 *P. dactylifera* (date palm) genotypes. For the commercial kit methods and the standard CTAB method, the air-dried or frozen samples were initially cut into small pieces with scissors and placed in a 2-mL tube from the Qbiogene Fast-DNA kit (Carlsbad, CA) containing a 6-mm ceramic sphere and garnet matrix. A second 6-mm ceramic sphere was added on top of the tissue, followed by the lysis solution from the respective kits, and samples were homogenized in a BIO 101 FastPrep (Qbiogene) at oscillation speed 5.0 for 40 s. A second, single speed machine, the Disruptor Genie (Scientific Industries, Bohemia, New York), was assessed using the D² BioTechnologies, Inc DNA isolation kit (Atlanta, GA), with the same ceramic spheres and garnet matrix, but in a 1.5 mL tube, for 2 min.

D² BioTechnologies DNA X-tract Plus Procedure. The first kit tested was the newly introduced DNA X-tract Plus kit (D² BioTechnologies Inc). Solution 1 (600−700 μL) was added to each tube prior to homogenization to achieve lysis. Samples were centrifuged for 30 s at low speed (4000*g*), and 175 μL of solution 2 was added to the homogenate. Tubes were mixed by inversion and incubated at room temperature for 10 min. Samples were then centrifuged at 5000*g* for 2 min, and the supernatants (~500 μL) were transferred into separate microcentrifuge tubes. An equal volume of chloroform was added to the supernatant and made homogeneous by vigorous inversion. Samples were centrifuged at 12000*g* for 5 min, the aqueous phase (upper layer) was transferred to a new tube, and the chloroform extraction step was repeated. The upper aqueous phase (~375−500 μL) was transferred to a fresh 1.5-mL tube, 525−700 μL of precipitation solution was added according to the manufacturer's instructions, and the samples were incubated on ice for 30 min or longer. Centrifugation at 12000*g* for 15 min precipitated the DNA. The DNA pellet was washed in 1 mL of 70% ethanol, centrifuged at 12000*g* for 5 min, air or vacuumed dried, and resuspended in 200 μL of sterile water.

Samples processed with the Disruptor Genie followed the DNA X-tract Plus procedure, after the 2-min of homogenization.

Qiagen DNeasy Plant System Procedure. The second method of DNA extraction was modified from the commercial DNeasy Plant System (Qiagen, Inc., Valencia, CA). Lysis was achieved by the addition of 400 μL of warm (up to 65 °C) lysis solution, modified by the addition of 10 mg/mL polyvinyl polypyrrolidone (PVPP) prior to homogenization in the FastPrep homogenizer. Samples were centrifuged 30 s at low speed (4000*g*), and 4 μL of RNase A stock solution (100 mg/mL) was added to each tube, followed by mixing until no tissue clumps were visible. The mixture was incubated for 20 min at 65 °C, and the tubes were inverted two or three times during incubation. Detergent, proteins, and polysaccharides were precipitated by the addition of 130 μL of buffer AP2 to the lysate, and the mixture was incubated on ice for 5 min. The ceramic beads were removed from the tubes with the bent tip of a spatula or "triceps" tweezers, and the dense solution was decanted and scraped into a QIAshredder spin column sitting in a 2-mL collection tube. The column−tube assembly was centrifuged for 2 min in a microcentrifuge at maximum speed. The volume of flow-through fraction was determined, and this liquid was transferred to a clean tube. Buffer AP3 (0.5 volume) and 100% ethanol

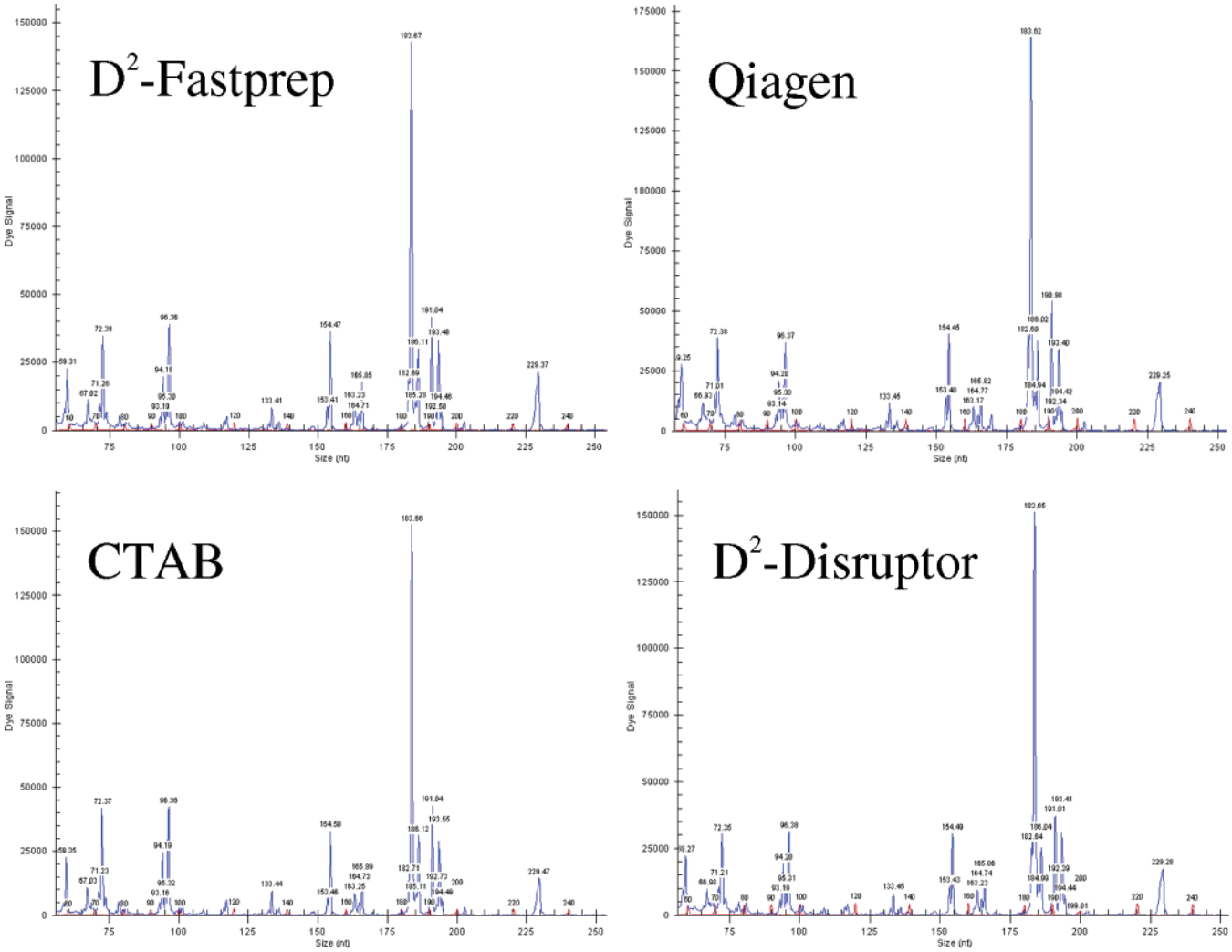

Figure 2. AFLP electropherograms of *P. dactylifera* (date palm) resulting from selective amplification with primers E-ACA/M-CAA and separation by capillary electrophoresis system. The four extraction procedures were D² BioTechnologies DNA FastPrep, Qiagen DNeasy, and traditional CTAB procedures, all with the FastPrep homogenizer; and the D² BioTechnologies procedure with the Disruptor Genie homogenizer. Internal DNA size standards are the tiny regular peaks (60, 70, 80, 90, 100, 120, ..., 240) in each electropherogram.

(1 volume) were added to the cleared lysate, and the solution was mixed by pipetting. DNA was bound to the DNeasy spin column by applying up to 650 μL of this sample mixture, including any precipitate that formed, into the column (in a 2-mL collection tube). The column−tube assembly was centrifuged for 1 min at 6000−12000*g*, and the flow-through was discarded. The remaining sample mixture was applied to the same column, the centrifugation step repeated, the flow-through discarded, and the collection tube replaced by a clean 2-mL tube. The column was washed at least twice by adding 500 μL of buffer AW (containing ethanol) onto the DNeasy column, centrifuging for 1 min at 6000*g* or more, and discarding the flow-through. Following the final wash, the column−tube assembly was centrifuged for 2 min at 10000−12000*g* to dry the column membrane, and the collection tube was discarded. The DNeasy column was transferred to a 1.5-mL microcentrifuge tube, and DNA was eluted from the membrane by pipetting 100 μL of preheated (65 °C) buffer AE directly onto the DNeasy column membrane, followed by incubation for 5 min at room temperature and then centrifugation (1 min at 6000*g* or greater). The elution step was repeated, yielding a final volume of 200 μL of DNA solution.

CTAB Procedure. The CTAB procedure was the modified method of Haymes (*12*). Tissue samples were ground to a fine powder in liquid nitrogen using a mortar and pestle. The resulting powder was mixed with 2% extraction buffer [100 mM Tris-HCl, pH 8.0; 1.4 M NaCl; 20 mM EDTA; 2% CTAB (hexadecyltrimethylammonium bromide, Sigma Chemical Co., MO); and 0.4% β-mercaptoethanol, added just before use] in the mortar and then transferred to a 1.5-mL microcentrifuge tube. Tubes were vortexed briefly, incubated in a 65 °C water bath for 1 h, and then allowed to cool to room temperature. After the addition of 0.8−1.0 mL of $CHCl_3$/isoamyl alcohol per tube, each tube was shaken vigorously to form an emulsion and then centrifuged at maximum speed (14000*g*) for 5 min. The aqueous phase (top layer) was then transferred to a clean 1.5-mL tube and was re-extracted with $CHCl_3$/isoamyl alcohol if it was still cloudy instead of clear. When the aqueous phase was clear, the DNA was precipitated by the addition of 1.0 mL of 95% ethanol. Tubes were kept on ice and not mixed until the DNA had begun to precipitate into the alcohol phase, after which the tubes were gently inverted three to four times, placed back on ice for 10 min, and then centrifuged at 14000*g* for 5 min. The pelleted DNA was washed in 70% ethanol. After the final wash treatment, the DNA was dried and then resuspended in 100 μL of sterile water. DNA samples were RNased at 37 °C for 60 min.

Quantification of DNA Samples. The presence of DNA was verified by analyzing 10-μL samples on a 1% TBE agarose gel supplemented with SYBR Green I (1:10000, Molecular Probes Inc., Eugene, OR). The quality and concentration of nucleic acid in the samples were evaluated spectrophotometrically, and double-stranded DNA concentration was determined by PicoGreen fluorescence kit (Molecular Probes, Inc.) using a Fluoroskan Ascent microplate reader equipped with 485/538 excitation/emission filter settings (LabSystems, Helsinki, Finland).

AFLP Analysis of Cacao and Date Palm. Techniques for AFLP analysis of cacao and date palm were adapted and modified from those published previously (*13*−*15*). Isolated DNAs were restricted with *Eco*RI and *Mse*I, and adapters were ligated to the restricted DNA fragments, using the materials and the protocol of the Life Technologies

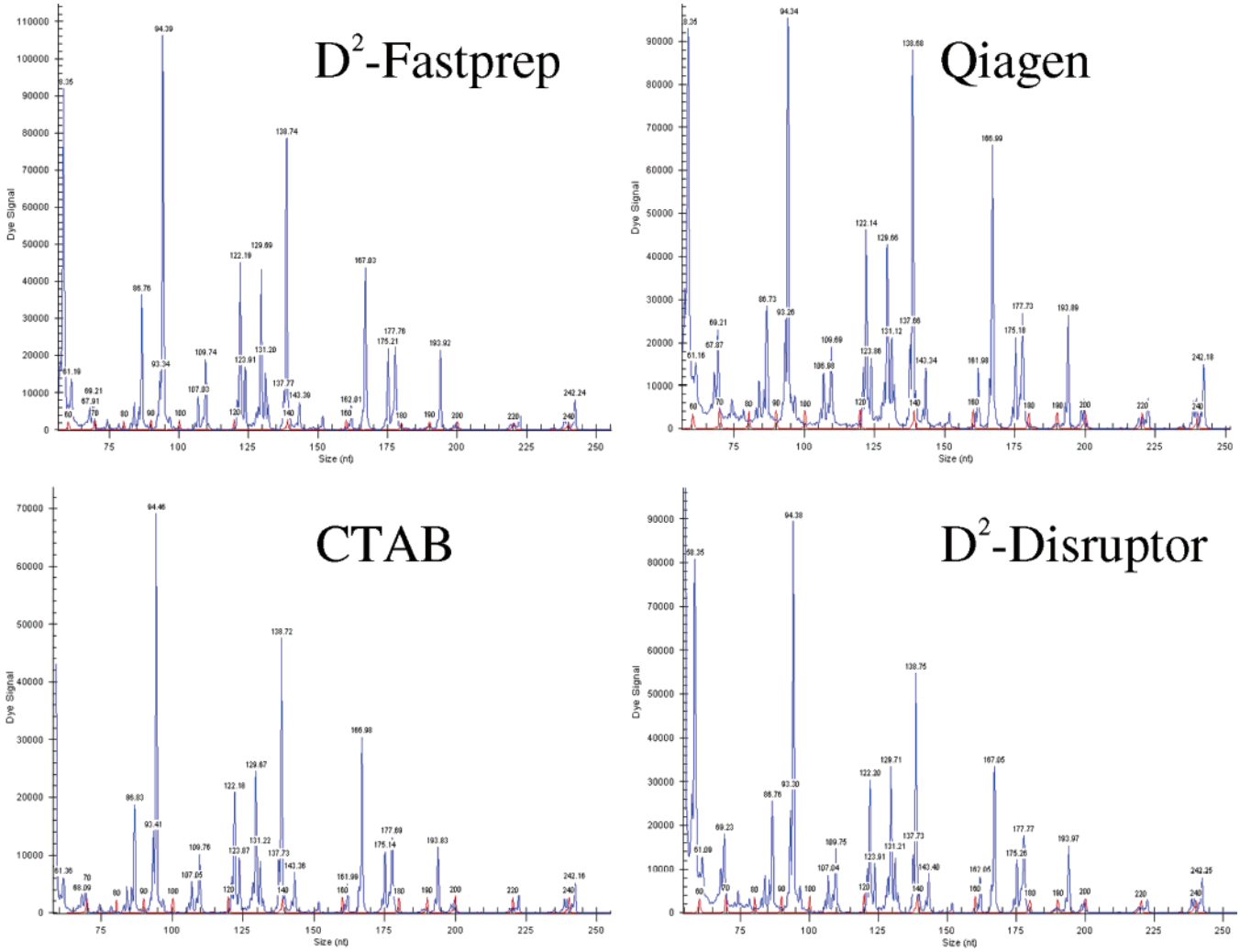

Figure 3. AFLP electropherograms of *T. cacao* (cacao) resulting from selective amplification with primers E-ACT/M-CAT and separation by capillary electrophoresis system. The four extraction procedures were D² BioTechnologies DNA FastPrep, Qiagen DNeasy, and traditional CTAB procedures, all with the FastPrep homogenizer, and the D² BioTechnologies procedure with the Disruptor Genie homogenizer. Internal DNA size standards are the tiny regular peaks (60, 70, 80, 90, 100, 120, ..., 240) in each electropherogram.

(Gaithersburg, MD) AFLP Analysis System I kit. Primers and *Taq* polymerase were used to amplify the preselective target fragments using a GeneAmp PCR System 9700 (PE Applied Biosystems, Foster City, CA) with the PCR program: 20 cycles of 94 °C for 30 s, 56 °C for 60 s, 72 °C for 60 s. An AFLP amplification core mix (Applied Biosystems) was used to select fragments for a second selective PCR amplification of 35 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 60 s, followed by a final extension period of 72 °C for 10 min. Forward selective primers (*Eco*RI) were synthesized by Research Genetics, Inc. (Huntsville, AL) and 5′ end-labeled with WellRED dyes (Beckman Coulter Inc., Fullerton, CA), and unlabeled reverse selective primers (*Mse*I) were synthesized by Operon (Alameda, CA). The selective primers used in this study were chosen on the basis of previous studies of successful AFLP primers in other crops (*2*). The forward primers were E-ACA (5′-GACTGCGTACCAATTCACA-3′) and E-ACT (5′-GACTGCGTACCAATTCACT-3′). M-CAA (5′-GATGAGTC-CTGAGTAACAA-3′) and M-CAT (5′-GATGAGTCCTGAGTAACAT-3′) were used as the reverse primers. For subsequent studies (data not shown) additional AFLP primers have been used on cacao DNAs extracted by the preferred methods with good results.

Samples were run individually on a CEQ2000XL eight-channel capillary electrophoresis DNA Analysis System (Beckman Coulter Inc.) to determine the size of the DNA fragments produced during the selective amplification step. The capillary injection consisted of a 30-s electrophoresis at 2.0 kV from a mixture of 0.3 μL of CEQ DNA size standard-400 (Beckman Coulter, Inc.), 1.5 μL of PCR amplification DNA fragments, and 30 μL of CEQ sample loading solution containing deionized formamide. The CEQ 2000 Frag-3 profile was used for the running conditions of capillary temperature = 50 °C, denaturation temperature = 90 °C for 120 s, and separation voltage of 6.0 kV for a run time of 35 min. Data analysis was performed using the CEQ 2000 Fragment Analysis software according to manufacturer's recommendations (Beckman Coulter Inc.).

SSR Analysis of Chocolate and Date Palm. Primer sequences for the analysis of microsatellites in *T. cacao* genomic DNA have been described by Lanaud et al. (*16*). The primer shown in this analysis was designed to amplify a dinucleotide microsatellite (GenBank locus TCA271942). The forward sequence was 5′-CTGGGTGCTGATAGA-TAA-3′, and the reverse sequence was 5′-AATACCCTCCACACAA-AT-3′). Other SSR primers tested are documented in GenBank with accession numbers Y16988, Y16980, and Y16982. Primer sets were synthesized and, as previously described, forward primers were labeled with a WellRED fluorescent dye by Research Genetics, Inc. Reaction mixtures consisted of 3 μL of diluted (1:100) genomic DNA template, 0.5 μL of each primer, and 20 μL of Platinum PCR SuperMix (Life Technologies), which contained an additional 30 units/mL of Platinum *Taq* polymerase (Life Technologies). Amplification was performed with the GeneAmp PCR System 9700 with the following profile: 95 °C for 5 min; followed by 35 cycles of 94 °C for 30 s, either 46 or 51 °C for 1 min (depending upon the annealing temperature of the primer pair), 72 °C for 1 min; followed by a hold at 60 °C for 15 min.

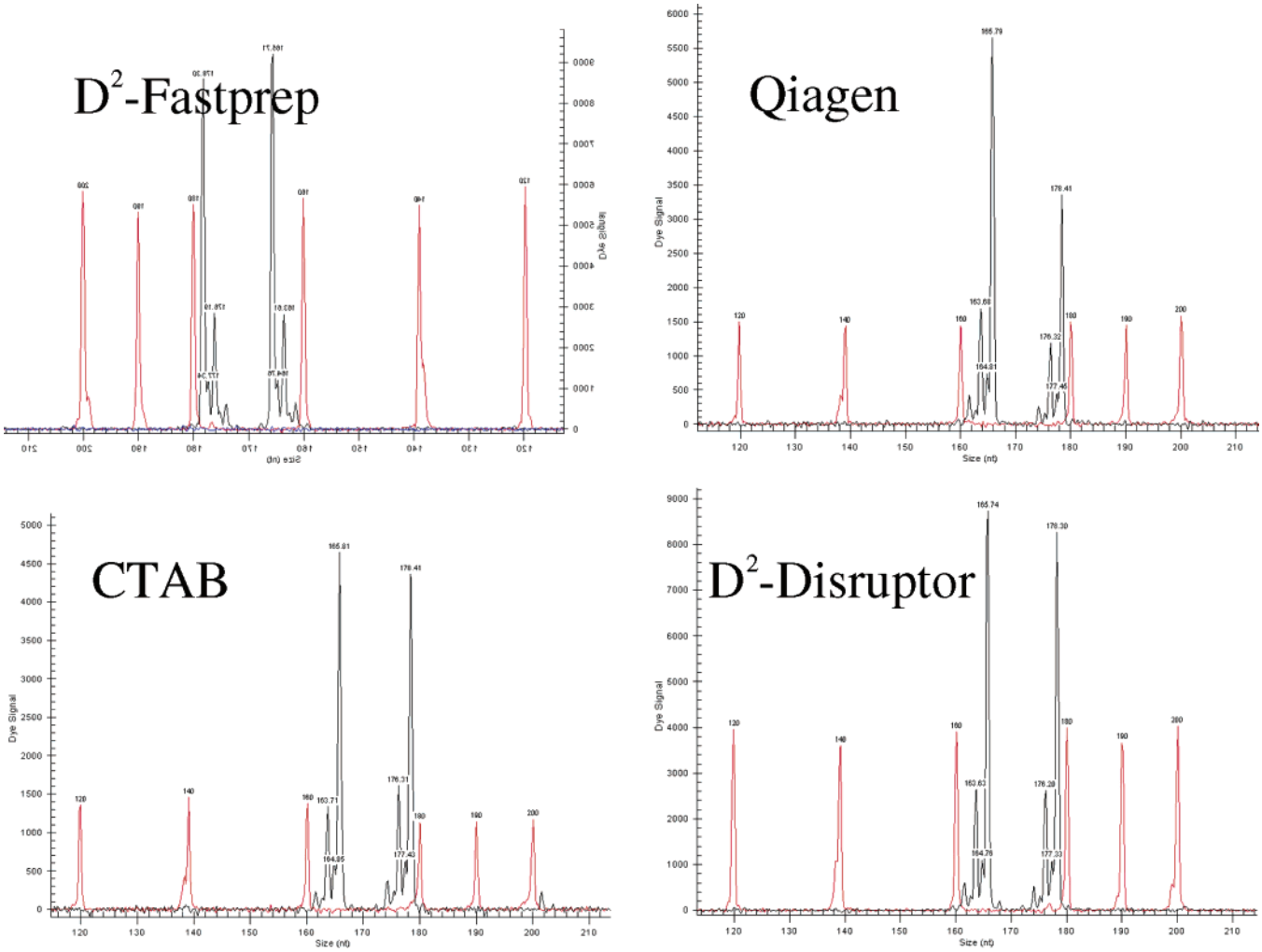

Figure 4. Electropherograms of microsatellites of *T. cacao* (chocolate tree). Primers amplifying locus TCA271942 were used for SSR analysis of cacao leaf tissue, and fragments were separated by capillary electrophoresis. The presence of two major peaks at 166 and 178 bp indicates the sample is heterozygous for the alleles at this locus, which differ by six dinucleotide repeats. The four extraction procedures were D² BioTechnologies DNA FastPrep, Qiagen DNeasy, and traditional CTAB procedures, all with the FastPrep homogenizer, and the D² BioTechnologies procedure with the Disruptor Genie homogenizer. Internal DNA size standards are the smaller regular peaks (60, 70, 80, 90, 100, 120, ..., 240) in each electropherogram.

The amplified microsatellite loci were separated by capillary electrophoresis and analyzed as described above on a CEQ2000XL capillary electrophoresis DNA Analysis System.

Three additional SSR primers that had amplified loci for cacao were tested to see if they would amplify in *P. dactylifera*. The PCR and analysis were performed in the same manner as stated above.

RESULTS AND DISCUSSION

Although PCR analysis does not always require DNA of the highest quality for successful amplification, a limiting step in the success of some DNA analysis procedures, such as AFLP, can be the quality of the DNA template. The initial step of this procedure requires digestion of the genomic DNA with restriction endonucleases, a process that may be inhibited by contaminants in the DNA extract. We have observed that isolation of genomic DNA by some protocols led to inconsistent results in AFLP analysis of plants (data not shown). Although a few investigations have focused on false-positive PCR results, false-negative results attributed to DNA polymerase inhibitors such as polysaccharides and phenolic compounds are common in some plants.

Protocols have been described that overcome the problem of inhibitory substances in plant tissues that interfere with PCR amplifications (*12*, *17*−*19*). The solutions in the D² BioTechnologies DNA X-tract Plus kits have been optimized for the recovery of DNA, without using a binding matrix, from fungi and plant tissues that are high in contaminants such as phenols and polysaccharides. The use of mechanical cell disruption devices, such as the BIO 101 FastPrep homogenizer or the Disruptor Genie, to break open resilient plant cell walls without extensive shearing of DNA, is very helpful in this process.

DNA suitable for molecular analysis was successfully isolated from mature leaves or regenerated shoot tissue of date palm or from fresh, frozen, or dried leaf tissue of *T. cacao* using both the D² BioTechnologies and the Qiagen DNA isolation procedures. In each case, the protocol was coupled with the use of a short mechanical disruption. With these techniques, only relatively small quantities of tissue were needed (25−50 mg) because excessive tissue decreased the efficiency of the process. DNA was also recovered with each of these procedures when the plant tissue was pulverized with a mortar and pestle in liquid nitrogen (data not shown); however, the mechanical homogenizers were a labor-saving convenience. Because the use of disposable tubes and grinding matrix completely prevented cross-contamination between samples, this alternative was economically advantageous.

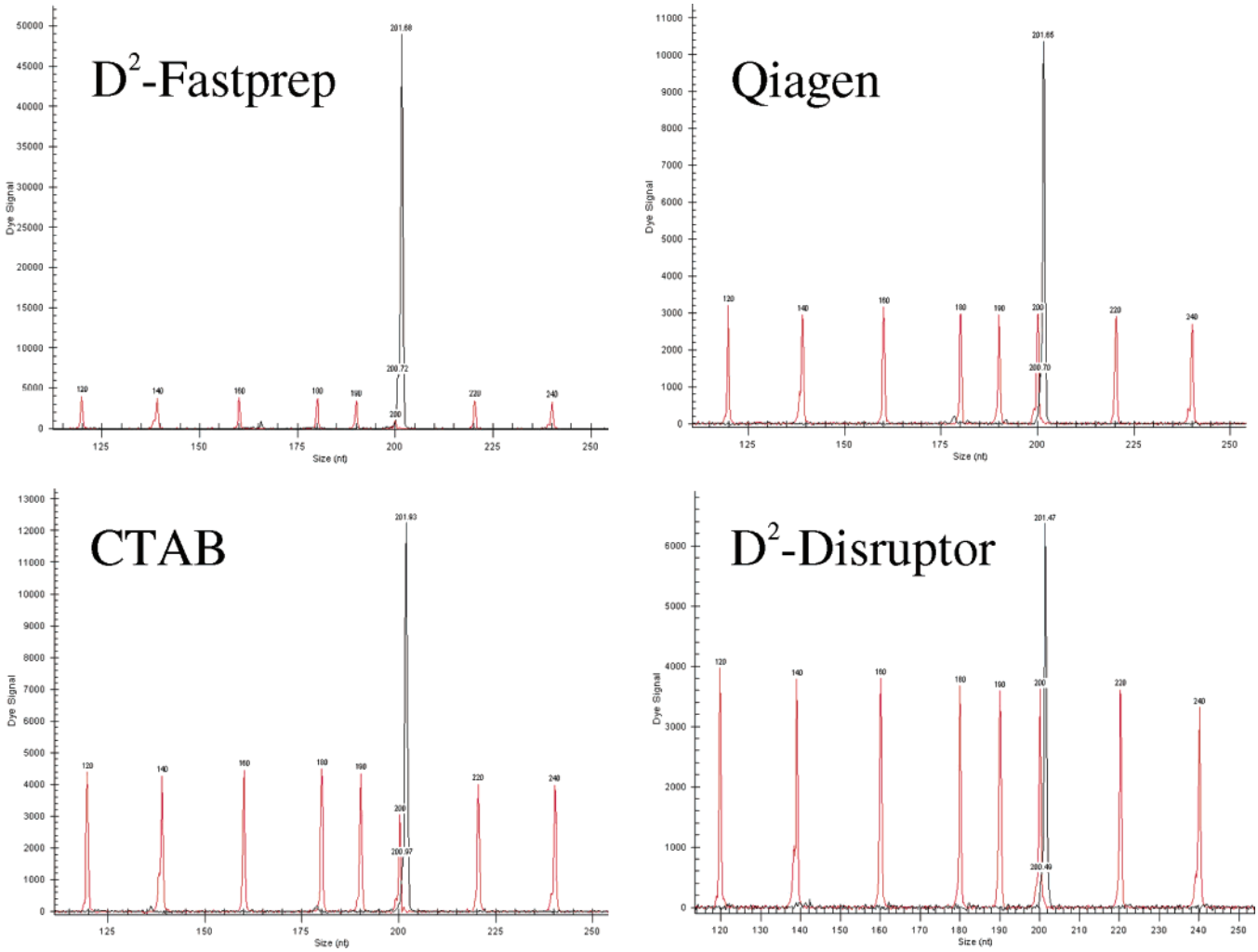

Figure 5. Electropherograms of amplified fragments generated by PCR with cacao SSR primers for locus TCA271942 and template DNA from *P. dactylifera* (date palm), separated by capillary electrophoresis. A single major peak at 201–202 bp demonstrates homologous sequences for primers in date palm. The four extraction procedures were D² BioTechnologies DNA FastPrep, Qiagen DNeasy, and traditional CTAB procedures, all with the FastPrep homogenizer, and the D² BioTechnologies procedure with the Disruptor Genie homogenizer. Internal DNA size standards are the smaller regular peaks (60, 70, 80, 90, 100, 120, ..., 240) in each electropherogram.

Date palm and chocolate DNAs isolated from equivalent samples by different extraction procedures, both using the FastPrep homogenizer, were quantitated by the PicoGreen DNA fluorescence, and the results are shown in **Figure 1**. This demonstrates that both the D² BioTechnologies and the Qiagen procedures were capable of isolating sufficient DNA from these species for further molecular characterization or analysis. Typically, 1−4 μg of DNA was extracted from either date palm or cacao using the strategies described.

AFLP analysis of DNA is often used when preliminary genomic sequence data or primers have not been developed for a species. Fragment analysis of a typical date palm AFLP profile by capillary electrophoresis instrument is shown in **Figure 2** for the CTAB and Qiagen DNA extraction protocols with the FastPrep homogenizer and for the D² BioTechnologies DNA extraction protocol with the FastPrep or the Disruptor Genie. DNA samples from each of these isolation protocols were successfully amplified and resulted in scorable AFLP profiles in date palm. Cacao was similarly assessed by AFLP (**Figure 3**). Repeated AFLP analyses demonstrated that any of these DNA isolation procedures, and either homogenizer, yielded DNA that was equally amenable for AFLP.

Another fingerprinting method of considerable interest is microsatellite analysis. Cacao DNA samples prepared from these isolation procedures were subjected to SSR analysis, using primers specifically developed for *T. cacao* (*16*). In separate tests, the microsatellite loci TCA16988, TCA16980, TCA16982, and TCA271942 were successfully amplified with primer sets Y16988, Y16980, Y16982, and AJ271942, respectively. **Figure 4** is a typical example, demonstrating the identical SSR profiles for the cacao sample when DNA was isolated with the different procedures.

Microsatellite amplification with the cacao primers in different species is theoretically difficult or impossible due to sequence divergence and ploidy variation, which affects the primer-annealing regions as well as the length of the SSR motifs themselves. However, it has been previously shown that cross-species amplification can occur (*20*−*22*). Thus, even though the microsatellite primers were designed for cacao, we tested them with date palm samples. The cacao SSR primer pair for locus TCA271942 resulted in successful amplification in date palm. As seen in **Figure 5**, the length of the fragment for date palm is ∼201 bp compared to the expected range in cacao of 165−185 bp (**Figure 4**). Additionally, each of the date palm samples we assessed was a different cultivar, and they all had the same fragment size (data not shown). Therefore, even though we observed successful amplification utilizing this microsatellite primer pair, the data were not useful. However, the purpose of

this study was not to distinguish the various date palm taxa but to show that the DNA obtained from the various methodologies could be amplified for SSR analysis. A recent study has been published that describes primers for SSR markers in *P. dactylifera* that were useful in other palm species (*23*).

Although the simplicity of scoring the SSR electropherograms makes them a method of choice in the selection of suitable DNA fingerprinting protocols, most plant species have not yet been subjected to detailed molecular characterizations to develop useable SSR primers. The use of AFLP DNA characterization in such species allows rapid, detailed information on the genetic diversity of a population without an in-depth project to first identify and sequence SSR repeating units and flanking regions.

In a comparison of the four methodologies, the CTAB procedure was the most labor intensive because all solutions needed to be prepared, and it also required the greatest amount of time to isolate samples. In studies involving thousands of samples, a protocol that is faster and less labor intensive is desirable. The D^2 BioTechnologies kit is a relatively low-cost DNA isolation procedure. For difficult plant samples, this kit yields DNA that is comparable to that obtained with other slower and more expensive procedures. The quantity and quality of DNA isolated by the D^2 BioTechnologies protocol are adequate for successful DNA characterization using AFLP and SSR analysis. The two homogenizers (the FastPrep and the Disruptor Genie) yielded similar results. The DNA yield was typically somewhat greater with the FastPrep homogenizer, but the quality and performance of the DNAs in analysis were the same. Due to the significant cost difference between the homogenizers, it was surprising to discover that cacao and date palm samples were both homogenized effectively and similarly by both. This study demonstrates the usefulness of the several approaches for large-scale molecular studies on cacao and other difficult plant material.

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